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(71) Applicant (*for all designated States except US*): **CRUCELL HOLLAND B.V.** [NL/NL]; Archimedesweg 4, NL-2333 CN Leiden (NL).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **MARZIO, Giuseppe** [IT/NL]; A. Constandsestraat 2, NL-1097 HX Amsterdam (NL). **PAU, Maria, Grazia** [NL/NL]; Kloksteeg 29, NL-2311 SK Leiden (NL). **OPSTELTEN, Dirk, Jan, Elbertus** [NL/NL]; Merelstraat 121, NL-2352 VC Leiderdorp (NL). **UYTDEHAAG, Alphonsus, Gerardus, Cornelis, Maria** [NL/NL]; Park Arenberg 41, NL-3731 EP De Bilt (NL).

(74) Agent: **KLEIN, Bart**; Crucell Holland B.V., Archimedesweg 4, P. O. Box 2048, NL-2301 CA Leiden (NL).

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(54) Title: PRODUCTION AND OF VIRUSES, VIRAL ISOLATES AND VACCINES

(57) Abstract: The present invention discloses methods for producing and/or selective propagating virus particles such as influenza virus particles preferably obtained from an infected subject by contacting and culturing a cell which overexpresses alpha-2,6 -or alpha-2,3 sialyltransferase with a virus particle under conditions conducive to propagation of said virus particle. The invention further provides the use of said virus particles for vaccine preparation.

Title: Production and of viruses, viral isolates and vaccines.

FIELD OF THE INVENTION

The invention relates to the field of medicine. The invention further relates to vaccines providing protection against influenza infection and to methods and means of obtaining these.

BACKGROUND OF THE INVENTION

Influenza viruses are the etiological agents of flu, a highly contagious respiratory illness that has afflicted humans since ancient times. The virus was first identified in 1933, but numerous epidemics almost certainly attributable to influenza were reported throughout the centuries (Potter 1998). There have been three major cases of outbreaks of influenza in the last century. The so-called 'Spanish flu' of 1918 was particularly severe. It resulted in the death of an estimated 20 to 40 million people worldwide, the most severe recorded outbreak of human disease known in history. In 1957 the 'Asian flu' killed an estimated 1 million people, and in 1968 the 'Hong-Kong flu' was lethal for more than 700,000 individuals. In spite of the efforts of the scientific community, infections caused by influenza viruses continue to claim each year a heavy toll in terms of cases of illness and death as well as economic consequences. Recent work has helped to explain the unusual virulence of some influenza strains that caused major pandemics in the past (Gibbs et al. 2001; Hatta et al. 2001). However, the understanding of the underlying pathogenic mechanisms is incomplete, thus limiting efficient prevention and treatment of the disease.

According to estimates that include all age groups, in the US alone 48 million persons suffer from flu each year. These epidemics result in approximately 20,000 deaths per year on top of about 4 million individuals that need treatment in a hospital (CDC statistics). Infants, children and the elderly are particularly susceptible to influenza infection. However, the

appearance of a new virus variant with high pathogenic and infective capacity remains a major threat to all individuals. This was proven to be the case in 1997, when a virus identified in Hong Kong caused the death of one third of the 18 clinically diagnosed cases (Claas et al. 1998; Subbarao et al. 1998).

Birds represent the major reservoir of influenza virus. In particular, all known subtypes of influenza A virus (together with subtype B the most common cause of flu in humans) have been isolated from wild- as well as domestic birds. However, an avian influenza A virus normally is not directly transmitted from birds to humans. In this respect, the only exception so far recorded has been the 1997 Hong Kong virus mentioned above. Several viral proteins are thought to play a role in conferring host specificity, but the most important factor is the hemagglutinin (HA) membrane protein.

The HA gene was one of the first genes of the influenza virus to be identified and sequenced. It codes for a trans-membrane protein directly involved in attachment to and penetration into the host cell. HA initiates infection by binding to terminal sialyl-oligosaccharide receptor determinants present on glycoproteins and gangliosides present on the host cell surface. Terminal sialic acid residues of natural sialyl-glycoproteins and gangliosides are known to be the minimum determinants of binding. However, binding depends also on the type of sialic acid linkage to penultimate galactose and on the structure of more distant parts of the sialyl-glycoconjugate.

Human influenza viruses bind preferentially to receptors containing the sialic acid alpha-2,6-galactose (SAalpha2,6Gal) linkage, whereas avian viruses use the SAalpha2,3Gal linkage (reviewed in Suzuki 1994). This binding specificity determines also the cell tropism of the virus inside the host. Human influenza virus infection (and replication) are restricted to the respiratory tract, whereas avian influenza virus is found mainly in the cells lining the intestinal tract as well as in the lungs of birds. Using sialic acid-galactose linkage specific lectins, it was shown that residues of sialic acid linked to galactose by the alpha-2,6 linkage but not SAalpha2,3Gal are present on the

surfaces of epithelial cells of the human trachea (Baum and Paulson 1990). Furthermore, also the abundance of SAalpha2,3Gal moieties in respiratory mucins contributes to maintain the SAalpha2,6Gal-specific phenotype of human influenza of HA (Baum and Paulson 1990; Couceiro et al. 1993).

In most laboratories propagation of primary isolates is still carried out in the chorio-allantoic sac of embryonated chicken eggs. This is due not only to historical reasons, but also to the lack of an appropriate alternative growth medium. This is currently also the system of choice for the production of large amounts of virus to be used in vaccine preparations. However, embryonated eggs have serious limitations as a host system for vaccine production. For instance, the lack of reliable year-round supplies of high-quality eggs as well as the limited availability of embryonated eggs in general may hamper vaccine production in case of the sudden outbreak of a new influenza subtype. Other disadvantages of this production system are the lack of flexibility, the risk of the presence of toxins and the risks of adventitious viruses, particularly retroviruses, and concerns about sterility.

Besides these limitations, culturing the virus on eggs poses a very significant additional problem, which is particularly important for vaccine purposes: There is now ample evidence that egg cultures lead to substrate-specific adaptation of the virus. In fact, even few passages in the allantoic sac of eggs are sufficient for a primary human isolate to adapt to the SAalpha2,3Gal binding phenotype (Rogers et al. 1985). This is due to the presence of SAalpha2,3Gal but not SAalpha2,6Gal residues on the cells lining the surface of the chicken embryo chorio-allantoic membrane. Virus variants present in primary isolates that are able to specifically interact with SAalpha2,3Gal residues have a replicative advantage over virus variants that interact more specifically to SAalpha2,6Gal residues. The SAalpha2,3Gal-specific virus variants are thus selected for in embryonated eggs (Gambaryan et al. 1999; Gambaryan et al. 1997). Egg-adaptation not only increases the affinity for SAalpha2,3Gal, but it also results in decreased affinity for

SAalpha2,6Gal. HA in fact cannot accommodate both types of analogues equally well, and multiple mutations have been identified that confer this altered binding specificity (Daniels et al. 1987; Gambaryan et al. 1999; Ito et al. 1997; Suzuki et al. 1989). Given the importance of HA in eliciting a specific immune response, these mutations result in major alterations of its antigenic properties (Ilobi et al. 1994; Robertson et al. 1994). Consequently, immunization with vaccines containing HA molecules bearing egg-induced mutations induces less neutralizing antibody to wild type influenza strains at the expenses of the level of protection achieved (Newman et al. 1993).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Schematic representation of pAlpha2,6ST2000/Hygro.

Figure 2. Schematic representation of (A) pAlpha2,6STcDNA2000/Neo and (B) pAlpha2,6STcDNA2000/Hygro.

Figure 3. Schematic representation of (A) pAlpha2,3STcDNA2000/Neo and (B) pAlpha2,3STcDNA2000/Hygro.

Figure 4. Detection of (A) SAalpha2,6Gal and (B) SAaplha2,3Gal in PER.C6 and PER.C6/alpha2,6ST by FACS analysis.

Figure 5. Propagation of a primary clinical influenza isolate and a egg-passaged influenza batch (from the same primary isolate) on PER.C6 and PER.C6/alpha2,6ST, determined by fluorescence. Infectivity is expressed as percentage of cells positive for HA-immunofluorescent staining.

Figure 6. Propagation of a primary clinical influenza isolate and a egg-passaged influenza batch (from the same primary isolate) on PER.C6 and PER.C6/alpha2,6ST, determined by plaque assay. Infectivity is expressed as plaque-forming units (pfu's) per ml.

SUMMARY OF THE INVENTION

The present invention discloses methods for producing and/or propagating virus particles such as influenza virus particles that preferably are present in a virus isolate obtained from an infected subject, said method comprising the steps of: contacting a cell with a virus particle and culturing said cell under conditions conducive to propagation of said virus particle, wherein said cell over-expresses a nucleic acid encoding an alpha2,6 or an alpha2,3 sialyltransferase. The invention also provides a method for selective propagation of a set of virus particles such as influenza virus particles present in an influenza isolate, wherein said set of virus particles has affinity for receptors comprising a specific glycosylation residue, said method comprising the steps of: incubating a cell with said isolate; culturing said cell under conditions conducive to propagation of said virus particle; and harvesting virus particles so produced from said cell and/or said culture medium.

The invention further provides novel vaccines and methods for making such vaccines, wherein said methods preferably comprise the steps of: treating the produced virus particles to yield antigenic parts; and harvesting at least one antigenic part such as hemagglutinin and/or neuraminidase from influenza virus. The invention further provides cells and cell lines and the use thereof, that over-express certain proteins involved in glycosylation for the production of vaccines, e.g, vaccines against influenza infection. Cells of the present invention are preferably human and transformed by adenovirus E1, such as PER.C6 cells or derivatives thereof.

DETAILED DESCRIPTION

The present invention provides methods for producing and/or propagating a virus particle, said method comprising the steps of: contacting a cell with a virus particle in a culture medium under conditions conducive to infection of said cell by said virus particle; and culturing said cell under conditions conducive to propagation of said virus particle, wherein said cell over-expresses a nucleic acid encoding an alpha2,6 sialyltransferase or a functional equivalent thereof. Said nucleic acid may encode an alpha2,6 sialyltransferase from different sources, such as rat and human. Preferably said alpha2,6 sialyltransferase is human alpha2,6 sialytransferase. The invention further provides methods for producing and/or propagating a virus particle, said method comprising the steps of: contacting a cell with a virus particle in a culture medium under conditions conducive to infection of said cell by said virus particle; and culturing said cell under conditions conducive to propagation of said virus particle, wherein said cell over-expresses a nucleic acid encoding an alpha2,3 sialyltransferase or a functional equivalent thereof. Said nucleic acid may encode an alpha2,3 sialyltransferase from different sources, such as rat and human. Preferably said alpha2,3 sialyltransferase is human alpha2,3 sialytransferase. In one embodiment of the invention, said virus particle is an influenza virus particle. Other non-limiting examples of virus particles that can be produced and/or propagated by using methods of the present invention are parainfluenza virus, Adeno-Associated virus (AAV) or poliomavirus. Any virus that utilizes the glycosylation structures that are induced by the alpha2,3 and alpha2,6 sialyltransferases can be propagated and/or produced by using methods of the present invention.

In a preferred embodiment the invention provides methods for propagating an influenza virus particle, wherein said influenza virus particle is present in an influenza isolate. More preferred are methods, wherein said influenza isolate is obtained from at least one influenza-infected mammalian

subject. Even more preferred are methods for propagating an influenza virus particle, wherein said influenza-infected mammalian subject is human or pig. In another embodiment the invention provides methods for producing and/or propagating an influenza virus particle, wherein said influenza isolate is obtained from at least one influenza-infected bird. Isolates as used herein refers to batches of influenza viruses that are obtained from subjects that are infected with influenza viruses. These subjects may be all species that are susceptible for influenza viruses, such as humans, birds, pigs and horses. Humans can get infected with influenza in different ways: either directly from other humans or directly from animal subjects such as pigs and birds. Propagated viruses that are used for vaccine manufacturing might be originally derived from one or more subjects (one or more human individuals, or one or more birds, pigs, etc.). In the case wherein influenza virus transmission from a bird to a human causes direct disease in humans, as was the case in the Hong Kong in 1997 (see above) it might be useful to be able to produce and/or propagate the influenza virus particles present in the bird isolate directly for vaccine manufacturing. The present invention provides methods for producing and/or propagating influenza virus particles present in isolates that are obtained from species such as birds, pigs, horses and humans by over-expressing the sialyltransferase proteins that are involved in the glycosylation of cell surface proteins and that generate the so-called SAalpha2,3Gal and SAalpha2,6Gal linkages in the oligosaccharide chains. Isolates as used herein preferably refers to clinical isolates (i.e., isolates obtained from diseased patients). Such clinical isolates are also referred to as primary isolates. Primary isolates can be influenza isolates directly obtained from for instance the nose, mucus and/or faeces of humans or animals that are infected with influenza virus(es). However, isolates that have been propagated on eggs on or cells or on other systems can still be further produced and/or propagated by methods of the present invention. Therefore, virus particles that are produced and/or propagated using the present invention may be present in

passaged batches, but are preferably present in primary batches, such as clinical isolates.

In a preferred embodiment of the invention the production and/or propagation of influenza virus particles is carried out by using cells in a culture medium, wherein said cell is transformed with E1 from adenovirus. More preferably, said cell is a human cell. In a highly preferred aspect, the invention provides methods for propagating an influenza virus particle according to the invention, wherein said human cell is PER.C6 or a derivative thereof.

PER.C6 cells are found to be useful for the propagation of different kinds of viruses such as rotavirus and influenza virus (see WO 01/38362). PER.C6 cells were first generated by transforming cells obtained from an embryonal retina with the E1 region of Adenovirus serotype 5. It was found that both alpha2,3 and alpha2,6 sialyltransferase proteins are present and active in PER.C6 cells (Pau et al. 2001). Therefore virus particles that specifically interact with the sialic acid – galactose linkage of the 2,3 type as well as of the 2,6 type (SAalpha2,3Gal and SAalpha2,6Gal respectively) were able to grow on PER.C6 cells. It is an important aspect of the invention that over-expression of either one of these sialyltransferase proteins leads to a specific propagation of sets of influenza viruses that either prefer the SAalpha2,3Gal residue or the SAalpha2,6Gal residue. This enables one to generate virus batches for vaccine production that have the best content for optimal protection. This content may differ. As discussed above, some spreading of the virus occurs mainly through human-human contact, while in others (such as the 1997 Hong Kong case, a direct bird-human contact was enough to sort a dramatic effect in humans. Depending on the virulence and the types of influenza viruses that play a role in this, a choice can be made for which set of virus particles in an isolate should be propagated with which the final vaccine is produced.

The present invention also provides methods for producing and/or propagating an influenza virus particle, wherein said nucleic acid encoding the sialyltransferase is heterologous to said cell. Preferably, said nucleic acid encoding the sialyltransferase is integrated into the genome of said cell. Heterologous as used herein means that the nucleic acid is manipulated such that the gene encoding the sialyltransferase expresses more of the protein than without said manipulation. Heterologous also means that the nucleic acid may be from a species that is different from the species from which the cell was derived, but may also be from the same species. A cell is said to over-express the sialyltransferase when the cell expresses more sialyltransferase than typical for that cell. A cell that over-expresses the sialyltransferase may also over-express the protein by manipulation of the genome of said cell such that the gene present in the genome of said cell expresses more of the protein than said cell did before it was manipulated. The over-expression may be induced by external means such as integration of a different or more-active promoter, by removal or inhibition of suppressors that normally limit the expression of the protein, or by chemical means. The over-expression may also be selected for. If cells are selected for a significant over-expression of at least one sialyltransferase they may be used for methods according to the present invention. Therefore, such cells and the use of such cells is also part of the present invention.

In another embodiment, the present invention provides methods for making a vaccine, said method comprising the steps of: producing and/or propagating a virus particle according to methods of the invention; and inactivating the virus particles so produced. Preferably said methods for making a vaccine further comprise the steps of: treating said virus particles so produced to yield antigenic parts; and obtaining at least one of said antigenic parts, preferably through means of purification and/or enrichment for said at least one part. Preferably a purified and/or enriched composition comprising said at least one obtained antigenic part does not comprise other antigenic

parts of said treated virus particles. In a more preferred embodiment the invention provides methods for making a vaccine, wherein said antigenic part comprises the hemagglutinin protein or a part thereof, and/or the neuraminidase protein or a part thereof from influenza virus. The neuraminidase (NA) and the hemagglutinin (HA) proteins are the most prominent antigenic parts of the influenza virus particle and are prone to differences during different propagation steps. The invention also provides vaccines obtainable according to methods of the present invention, while it also provides pharmaceutical compositions comprising a vaccine obtainable according to the present invention.

As mentioned, the cells of the present invention are extremely useful for the propagation of primary, clinical isolates comprising influenza virus particles, while said cells can also be applied for propagating isolates that already have been passaged on embryonated eggs or on other systems, to obtain a selection of influenza virus particles that recognize specific glycosylation residues present on glycoproteins. Thus, the present invention also provides the use of a cell line over-expressing an alpha2,6 sialyltransferase or a functional part thereof for the propagation of a virus particle and the use of a cell line over-expressing an alpha2,3 sialyltransferase or a functional part thereof for the propagation of a virus particle. Preferably, said virus particle is an influenza virus particle. More preferably, said influenza virus particle is present in an influenza isolate obtained from at least one influenza-infected mammalian subject. Even more preferred are uses of said cell line according to the present invention, wherein said influenza-infected mammalian subject is a human or a pig, whereas it is also preferred that said influenza virus particle is present in an influenza isolate obtained from at least one influenza-infected bird.

The present invention further provides a method for selective production and/or propagation of a set of predetermined virus particles present in an isolate, wherein said set of predetermined virus particles has a preference for a

specific glycosylation moiety present on a receptor, and wherein said isolate comprises in addition to said set also virus particles not having said preference, said method comprising the steps of: incubating a cell which is capable of expressing and exposing said receptor comprising said specific glycosylation moiety, with said isolate in a culture medium under conditions conducive to infection of said cell by at least one virus particle present in said set; culturing said cell under conditions conducive to propagation of said virus particle; and harvesting virus particles so produced from said cell and/or said culture medium. A glycosylation moiety as used herein refers to any kind of residue, linkage and or group of sugar types present in a oligosaccharide chain on a glycoprotein that is recognized by a virus particle for infection. Preferably said glycosylation moiety comprises a SAalpha2,6Gal residue or a SAalpha2,3Gal residue. More preferred are methods wherein said set of predetermined virus particles is a set of predetermined influenza virus particles. The SAalpha2,6Gal residue and SAalpha2,3Gal residues are specifically recognized by the HA protein of the virus particle, in the case of influenza. It depends on the HA protein whether there is any specificity in the interaction with either one residue. In general, influenza isolates comprise viruses that interact specifically with the SAalpha2,6Gal residue as well as viruses that specifically interact with the SAalpha2,3Gal residue. With the present invention it is now possible to selectively propagate either set of viruses from clinical, primary and/or passaged isolates to obtain propagated sets of viruses that are useful in the production of an influenza vaccine, useful in humans. Besides the fact that vaccines can be produced for humans, it is also possible by using methods and means of the present invention to selectively propagate viruses for the manufacturing of veterinary applications to for instance prevent the spreading of influenza viruses through swine or horse populations. Preferably, said influenza isolate is obtained from at least one influenza-infected human, pig or bird. It is also preferred that said cell is a human cell and that it is transformed with E1 from adenovirus. Highly

preferred are cells that are PER.C6 cells or derivatives thereof. Derivatives as used herein refers to modified versions of the original PER.C6 cells, wherein for instance other heterologous nucleic acids are introduced, knocked-out, or in other ways modified. Non-limiting examples of PER.C6 derivatives are PER.C6 cells that stable express a temperature sensitive mutant of Adenovirus E2A, or that express other adenovirus nucleic acids such as E4. If certain nucleic acids in PER.C6 cells have been switched on or off by other means such as chemical treatment or knock-out techniques, these cells still remain PER.C6 derivatives. Although the examples provided describe the use of cells that over-express the erythropoietin (EPO) protein it should be noted that it is not a part of the invention to have over-expression of EPO in the cells of the invention.

In another preferred embodiment the invention provides methods for selective propagation of a set of virus particles present in an isolate, wherein said cell comprises a nucleic acid encoding a sialyltransferase that is heterologous to said cell. Even more preferred are methods according to the present invention, wherein said nucleic acid encoding a sialyltransferase is integrated into the genome of said cell. Such an integrated nucleic acid is preferably stably integrated through the use of selection markers such as the hygromycin and neomycin resistance genes.

The present invention also provides human cells comprising a heterologous nucleic acid encoding an alpha2,6 sialyltransferase or an alpha2,3 sialyltransferase. Preferably, said nucleic acid is integrated into the genome of said human cell. The invention also provides the use of such cells for the selective propagation of virus particles, preferably being influenza virus particles.

The present invention provides optimization of a process for propagation of primary isolates of human influenza virus. Also, the present invention provides optimization of a process for propagating primary as well as laboratory isolates of influenza viruses using the SAalpha2,6Gal or

SAalpha2,3Gal (or both) glycosylation moieties present on cell surface glycoproteins. In general human influenza viruses recognize the SAalpha2,6Gal moiety, while the avian influenza viruses recognize the SAalpha2,3Gal moiety. The swine influenza viruses generally utilize both residues. The invention provides optimization of a process for propagation of any virus for which the replication depends on the activity of alpha2,3 sialyltransferase and/or alpha2,6 sialyltransferase, or more generally, on the presence of SAalpha2,3Gal or SAalpha2,6Gal residues. The methods of the present invention comprise the use of cells in a culture medium. As an example of such a process, human cells were taken that are known to support efficient replication and production of influenza viruses.

The cells of the present invention are not only useful for the propagation of influenza viruses. It is well known in the art that other viruses such as Adeno-Associated Virus (AAV), human poliomavirus and parainfluenza viruses utilize the alpha2,3 and alpha2,6 linkages in glycoproteins for infection (Liu et al. 1998; Suzuki et al. 2001; Walters et al. 2001). Therefore the present invention also provides methods for (selective) production and/or propagation of other viruses that use these glycosylation structures for recognition and infection of the targeted cell. Furthermore, the invention provides the use of the cells of the invention and the methods and means for the production of viruses other than influenza and for the production of vaccines against such viruses, if applicable. The invention therefore also provides vaccines against viruses that utilize the SAalpha2,3Gal and the SAalpha2,6Gal residues for cellular recognition and infectivity.

It has been previously demonstrated that PER.C6™ cells (ECACC deposit 96022940) represent an ideal substrate for the propagation of influenza virus and that the production levels from PER.C6 resulted in high-titer preparations suitable for vaccine purposes (WO 01/38362). A novel cell line provided by the present invention, named 'PER.C6-alpha2,6ST' is derived from PER.C6 through the following process: a plasmid harboring a nucleic acid

encoding human alpha2,6 sialyltransferase under the control of the strong CMV promoter was transfected into PER.C6 cells and cells were subsequently selected for stable integration of the plasmid. The PER.C6-alpha2,6ST cells are characterized by the higher expression of SAalpha2,6Gal-containing receptors as compared to the number of receptors carrying the SAalpha2,6Gal residue in the original PER.C6 cells. This does not directly imply that the proteins carrying such moieties are over-expressed but that the percentage of proteins carrying the SAalpha2,6Gal residue is higher than the percentage of such proteins in PER.C6 cells. PER.C6 cells are without over-expression of the alpha2,6 sialyltransferase already capable of expressing both SAalpha2,3Gal and SAalpha2,6Gal residues on cell surface glycoproteins. It is however an important aspect of the present invention to increase the percentage of proteins carrying the SAalpha2,6Gal residue in comparison to the percentage of proteins that carry the SAalpha2,3Gal residue. Due to direct substrate competition in the intracellular glycosylation machinery, receptors of the SAalpha2,3Gal type become underrepresented on the cell surface of cells over-expressing the alpha2,6 sialyltransferase protein. These combined characteristics make this new cell line an ideal medium for propagating primary influenza virus isolates without inducing selection pressure in the wild type population. The propagation of such isolates on the cells of the present invention results in efficient production of large virus stocks with unaltered HA specificity and immunogenicity that are highly useful for the production of vaccines. As virus produced in PER.C6-alpha2,6ST does not present mutations resulting from adaptation to the SAalpha2,3Gal receptor (as is the case for embryonated eggs) the immunogenic properties of this virus are most comparable with those of naturally circulating influenza viruses. Consequently, vaccine preparations obtained from influenza virus grown on PER.C6-alpha2,6ST are ideally suited to induce a protective response against circulating wild type influenza virus. It is known in the art that human influenza viruses are of the type recognizing the SAalpha2,6Gal linkages and

it is therefore recognized in the art that it is desired to obtain vaccines comprising proteins from these viruses in order to sort a more protective immune response in humans (Newman et al. 1993).

If human influenza viruses are propagated via embryonated chicken eggs, virus variants that are able to bind specifically to SAalpha2,3Gal will be selected for, and the SAalpha2,6Gal recognizing viruses will be selected out. PER.C6 cells have both SAalpha2,6Gal and SAalpha2,3Gal containing receptors at its surface. For a preferred propagation of the SAalpha2,6Gal recognizing viruses it is therefore preferred to have over-expression of receptors that harbor this component, as discussed above. To determine the effect of the opposite, namely over-expression of human alpha2,3 sialyltransferase, the present invention provides also methods and means for generating another novel cell line named 'PER.C6-alpha2,3ST'. These cells are derived from PER.C6 in a similar manner as described above for the PER.C6-alpha2,6ST cells, by transfection of a plasmid harboring nucleic acid encoding human alpha2,3 sialyltransferase under the control of the strong CMV promoter, after which cells carrying a stable integration of the plasmid are selected. A PER.C6-alpha2,3ST cell is characterized by the higher expression of SAalpha2,3Gal-containing receptors.

Both alpha2,6 sialyltransferase and alpha2,3 sialyltransferase over-expressing cell lines are useful since alpha2,6 sialyltransferase over-expressing cells can be used for the propagation of influenza viruses that preferably recognize the SAalpha2,6Gal residue, while the alpha2,3 sialyltransferase over-expressing cells can be used for the propagation of influenza viruses that preferably recognize the SAalpha2,3Gal residue. When the infection and the spreading of the viruses mainly occurs via human-human contact and the viruses become more adapted to the infectious route via the SAalpha2,6Gal residues, then it is preferred to apply the alpha2,6 sialyltransferase over-expressing cell line. On the other hand, when the infectivity occurs directly from birds that do not have glycoproteins harboring

the SAalpha2,3Gal residue to humans (as was the case in the small but severe epidemic in Hong-Kong in 1997) then it is preferred to apply cells that over-express the alpha2,3 sialyltransferase.

As used herein the terms alpha2,3 sialyltransferase or alpha2,3 sialyltransferase refer to the respective transferases and also to equivalents of said transferase, wherein said equivalents comprise the same transferase activity in kind not necessarily in amount as the transferase it is equivalent to. Suitable equivalents can be generated by the person skilled in the art. A part of said transferase is a suitable equivalent if it comprises the same transferase activity in kind not necessarily in amount. Other suitable equivalents are derivatives and/or analogues of alpha2,3 sialyltransferase or alpha2,3 sialyltransferase comprising the same transferase activity in kind not necessarily in amount as the transferase it is equivalent to. Such derivatives may be generated through conservative amino acid substitution or otherwise. A derivative can also be made from a part of the respective transferases. An influenza virus particle as used herein can be an influenza virus or an influenza virus like particle. An equivalent of an influenza virus particle is a virus(like) particle comprising the same infectivity properties in kind not necessarily in amount as an influenza virus particle. Such equivalents can for instance be generated by recombinant means. Such equivalents may comprise molecules not typically present in an influenza virus.

EXAMPLES

Example 1. Construction of pAlpha2,6ST2000/Hygro.

The fragment containing the sequence coding for alpha2,6 sialyltransferase was obtained by EcoRI digestion of plasmid pGST-Gal (a gift from Dr. I. van Die, Free University of Amsterdam; The plasmid consists of a pBR322 backbone containing the entire cDNA sequence coding for rat alpha2,6 sialyltransferase, GenBank accession nr. M18769). The fragment was made blunt-ended by T4 DNA polymerase according to standard procedures. After gel purification, the alpha2,6 sialyltransferase encoding fragment was ligated into pcDNA2000/Hygro (also known as plasmid pcDNA2000/Hyg(-) which has been described in WO 00/63403), which was linearized with PmeI, dephosphorylated and gel purified according to standard laboratory procedures. The resulting plasmid was named pAlpha2,6ST2000/Hygro (Figure 1).

Example 2. Transfection of pAlpha2,6ST2000/Hygro in PER.C6-EPO and selection of overexpressing clones.

PER.C6-EPO were initially generated for other purposes, namely for experiments focusing on glycosylation of erythropoietin (EPO). EPO is a protein involved in stimulation of erythropoiesis and its activity depends heavily on its sialic acid content for in vivo functionality. The PER.C6-EPO cell line is a derivative of PER.C6 and overexpresses the human EPO protein (cells have been described in WO 00/63403). The fact that this cell line is producing EPO is not believed to be critical for the present invention. PER.C6-EPO cells were cultured and transfected with pAlpha2,6ST2000/Hygro as described below.

PER.C6 cells were seeded in tissue culture dishes (10 cm diameter) with approximately 2-3 million cells/dish and were kept overnight at 37°C and 10% CO_2. On the next day, cells are transfected using Lipofectamine (Gibco)

according to the manufacturer's protocol. Twenty dishes were transfected each with 2 µg of pAlpha2,6ST2000/Hygro all according to standard protocols well known to persons skilled in the art. Another 6 dishes served as negative controls for hygromycin killing and transfection efficiency. On the next day, hygromycin was added to the dishes at a concentration of 50 µg/ml, dissolved in DMEM medium containing FBS. Cells were incubated over a period of 3-4 weeks, with regular washing of the cells with fresh medium supplemented with hygromycin. Cells were monitored daily for death, comparing with the negative controls that did not receive the plasmids harboring the hygromycin selection markers. Outgrowing colonies were picked and subcultured generally as described for erythropoietin- and antibody-overexpressing cell lines in WO 00/63403. Approximately 25 selected antibiotic-resistent colonies were grown subsequently in 24-wells, 6-wells plates and T25 flask without hygromycin. When cells reached growth in T75 tissue culture flasks at least one vial of each clone was frozen and stored for back-up. The clones were subsequently tested for alpha2,6ST activity by FACS analysis on a FACsort apparatus (Becton Dickinson) using methods previously described by Govorkova et al. (1999). For this, the SAalpha2,6Gal-specific *Sambucus nigra* agglutinin (DIG Glycan differentiation kit, Roche) was used following the supplier's protocols. These clones were subcultured in a time span of two months, during which FACS analysis experiments were performed on a regular basis to monitor expression of alpha2,6 sialyltransferase on the cell surface. Increased expression of SAalpha2,6Gal was stable. The best alpha2,6 sialyltransferase-expressing clone, as assessed by the highest density of SAalpha2,6Gal on the cell surface, was clone 25-3.10. This clone was named 'PER.C6-alpha2,6 ST'. The results in Figure 4A show a FACS analysis on PER.C6-alpha2,6 ST at the end of the selection process. It is evident that stable transfection of pAlpha2,6ST2000/Hygro leads to markedly increased levels of SAalpha2,6Gal residues on the cell surface as compared to the maternal PER.C6 cell line. Interestingly, over-expression of alpha2,6 sialyltransferase also seems to result

in lower amounts of SAalpha2,3Gal residues, as detected by FACS using alpha2,3Gal-specific *Maackia amurensis* agglutinin (Figure 4B). This effect is most likely due to competition of alpha2,6 sialyltransferase with endogenous alpha2,3 sialyltransferase for the same glycoprotein substrate.

Example 3. Generation of alpha2,6- and alpha2,3 sialyltransferase cDNA expression vectors.

A PCR fragment containing the full length cDNA of human alpha2,6 sialytransferase (GenBank accession nr: 14735135) is obtained by Polymerase Chain Reaction (PCR) on a human cDNA library using methods well known to persons skilled in the art. The primers used for the amplification (sense: 5'-TTT TTT GGA TCC ATG ATT CAC ACC AAC CTG AAG AAA AAG-3', antisense: 5'-TTT TTT CTT AAG TTA GCA GTG AAT GGT CCG GAA GC-3') contain an additional 5'-tail that allows digestion with BamHI in the sense primer and AflII in the antisense primer respectively. The PCR product is purified via agarose gel electrophoresis and digested with BamHI and AflII and subsequently cloned into pcDNA2000/Hygro (described as pcDNA2000/Hyg(-) in WO 00/63403) and into pcDNA2000/Neo (this vector was basically constructed in the same way as pcDNA2000/Hyg(-) from pcDNA2000/DHFR as has been described in detail in WO 00/63403). For this, pcDNA2000/Hygro and pcDNA2000/Neo were also digested with BamHI and AflII restriction enzymes. The sequence and the correct cloning are checked by double stranded sequencing according to standard procedures known to persons skilled in the art of molecular biology. The resulting plasmids are named pAlpha2,6STcDNA2000/Hygro (Figure 2A) pAlpha2,6STcDNA2000/Neo (Figure 2B). They comprise nucleic acid encoding human alpha2,6 sialyltransferase under the control of the extended CMV promoter (see WO 00/63403). Furthermore, the plasmids confer resistance to neomycin and hygromycin respectively, that are used to select for clones that have integrated the plasmid into their genome in a stable manner.

The cDNA of human alpha2,3 sialyltransferase (GenBank accession nr. L23767) is obtained and cloned as described above for the human alpha2,6 sialyltransferase gene. The primers that are used for the PCR reaction are: sense 5'-TTT TTT GGA TCC ATG TGT CCT GCA GGC TGG AAG CTC-3' and antisense 5'-TTT TTT CTT AAG TCA GAA GGA CGT GAG GTT CTT GAT AG-3'. The resulting plasmids are named pAlpha2,3STcDNA2000/Hygro (Figure 3A) pAlpha2,3STcDNA2000/Neo (Figure 3B).

Example 4. Generation of stable PER.C6 cells over-expressing either human alpha2,6- or human alpha2,3 sialyltransferase.

Cells of the PER.C6 cell line are seeded in 40 tissue culture dishes (10 cm diameter) with approximately 2-3 million cells/dish and are kept overnight at 37°C and 10% CO_2. On the next day, cells are transfected using Lipofectamine (Gibco) according to the manufacturer's protocol and to standard culturing procedures known to persons skilled in the art. Twenty dishes are transfected each with 5 µg of pAlpha2,6STcDNA2000/Neo. Another 20 dishes with non-transfected cells serve as negative controls for neomycin killing and transfection efficiency. On the next day, neomycin (0.5 mg/ml) is added to the appropriate dishes, dissolved in medium containing FBS. Cells are incubated over a period of 4-5 weeks, with regular washing of the cells with fresh medium supplemented with the selection agent. Cells are monitored daily for death, comparing with the negative controls that did not receive the plasmids harboring the neomycin and hygromycin selection markers. Outgrowing colonies are picked and subcultured generally as described for erythropoietin- and antibody-overexpressing cell lines in WO 00/63403.

From each cell line, approximately 50 selected neomycin-resistent colonies are grown subsequently in 96-wells, 24-wells, 6-wells plates and T25 flask with neomycin. When cells reach growth in T25 tissue culture flasks at least one vial of each clone is frozen and stored for back-up. Each clone is subsequently tested for production of recombinant human alpha2,6

sialyltransferase by FACS analysis using SAalpha2,6Gal-specific *Sambucus nigra* agglutinin as described above and as previously described by Govorkova et al. (1999). The following selection of good producer clones is based on expression, culturing behaviour and viability. To allow checks for long term viability, suspension growth in roller bottles and bioreactor during extended time periods, more vials of the best performing clones are frozen, and are selected for further investigation. These clones are subcultured in a time span of two months. During these two months, FACS analysis experiments are performed on a regular basis to monitor expression of alpha2,6 sialyltransferase on the cell surface. The best stable producer is selected and used for cell banking. This clone is expanded to generate a cell line that is named PER.C6-H-alpha2,6 ST.

Cell lines over-expressing the human alpha2,3 sialyltransferase protein are generated in generally the same way as described above for the human alpha2,6 sialyltransferase over-expressing PER.C6 cells. In this case plasmid pAlpha2,3STcDNA2000/Neo is used. The resulting cell line is named PER.C6-H-alpha2,3 ST.

Example 5. Cell culture and infection with primary and adapted influenza virus isolates in PER.C6 cells and in alpha2,6 sialytransferase-overexpressing PER.C6 cells.

Experiments were performed to compare the susceptibility to infection of PER.C6 with that of PER.C6-alpha2,6 ST. Suspension cultures of PER.C6 and PER.C6-alpha2,6 ST were cultured in serum-free ExCell 525 medium (JRH Biosciences) supplemented with 4 mM L-Glutamin (Gibco), at 37°C and 10% CO_2 in 490 cm^2 tissue culture roller bottles during continuous rotation at 1 rpm. The procedure described below was applied for all the influenza infections reported. At the day of infection, cells were seeded in 6-well plates, at the density of $1x10^6$ cells/ml in a final volume of 2 ml of serum-free media, containing 2 mg/ml Pen/Strep (Gibco), 200 ng/ml Fungizone (Gibco) and 3

μg/ml trypsin-EDTA (Gibco). Cells were infected with a viral inoculum of a primary isolate and with a PER.C6-adapted batch (derived from the primary isolate and passaged for 1 passage on PER.C6 cells). The primary isolate that was used is the A/Netherlands/002/01 (H1N1, A/New Caledonia like, gift from Prof. Dr. A. Osterhaus, University of Rotterdam). Both batches were used at a 10^{-2} v/v dilution. Infected cells were kept in static culture at 35°C, in 10% CO_2, for six days. Viral supernatants were retrieved throughout the experiment and subsequently clarified. Clarification was performed by pelletting the cells in a microfuge at 5000 rpm for 5 min, at room temperature. Cell pellets were analyzed by direct immunofluorescence assay as described infra. Supernatants were transferred to a new eppendorf tube, rapidly frozen in liquid N_2 and stored at –80°C until use in plaque assays (see below).

Example 6. Immunofluorescence test.

Direct immunofluorescence (I.F.) assays for the detection of Influenza virus infection were carried out in infected cells (see above) using the IMAGEN™ Influenza Virus A and B kit (Dako) according to the protocol provided by the supplier. Briefly, infected cells were centrifuged for 5 min. The supernatant was removed and the pellet resuspended in PBS. This was repeated twice to wash the cells thoroughly. The washed cell pellet was resuspended in PBS and 20 μl of cell suspension was added to each of two wells of an I.F. slide. This was allowed to dry at room temperature. The cells were fixed by adding 20 μl acetone to each well and air-dried. To each well, 20 μl of the appropriate IMAGEN Influenza reagent (i.e., labeled antibody specific Influenza A or B) was added. The slide was then incubated for 15 min at 37°C on a damp tissue. Excess reagent was washed away with PBS and then rinsed for 5 min in PBS. The slide was air-dried at room temperature. One drop of IMAGEN mounting fluid was added to each well and a cover slip placed over the slide (this was fixed in place with a small amount of nail polish). Samples were viewed microscopically using epifluorescence illumination. Infected cells

were characterized by a bright apple-green fluorescence. The approximate percentage of cells that show positive (fluorescent green) compared with negative (red) cells was recorded. Results are shown in Figure 5. It is evident that PER.C6-alpha2,6 ST supported efficiently the replication of the clinical isolate (white bars).

Example 7. Plaque assay

Virus production in PER.C6 and PER.C6-alpha2,6 ST were studied by scoring for plaque formation in MDCK (Madin Darbin Canine Kidney) cells inoculated with virus supernatants. MDCK cells are particularly useful for such plaque assay experiments. A total of 1 ml of 10-fold diluted viral supernatants of primary and PER.C6-passaged influenza virus both propagated on PER.C6 and PER.C6-alpha2,6 ST according to the methods described in example 5, were inoculated on MDCK cells which were grown until 95% confluence in 6-well plates in DMEM supplemented with 2 mM L-glutamin. After 1 h at 37°C the cells were washed twice with PBS and overloaded with 3 ml of agarose mix (1.2 ml 2.5% agarose, 1.5 ml 2x MEM, 30 µl 200 mM L-Glutamine, 24 µl trypsin-EDTA, 250 µl PBS). The cells were then incubated in a humid, 10% CO_2 atmosphere at 37°C for approximately 3 days and viral plaques were visually scored and counted. Results are shown in Figure 6. The clinical isolate of influenza virus (white bars) and the PER.C6-passaged virus (grey bars) could infect the PER.C6-alpha2,6 ST cells very efficiently (right panel), whereas PER.C6 cells (left panel) were not very susceptible to infection by the primary clinical isolate. This shows that cells that over-express the alpha2,6 sialyltransferase are particularly useful to propagate primary virus isolates and shows that these cells are extremely useful in rapid and safe methods for the production of vaccines against for instance influenza infection.

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CLAIMS

1. A method for producing a virus particle, said method comprising the steps of:
   - contacting a cell with a virus particle in a culture medium under conditions conducive to infection of said cell by said virus particle; and
   - culturing said cell under conditions conducive to propagation of said virus particle,

   wherein said cell over-expresses a nucleic acid encoding an alpha2,6 sialyltransferase or a functional equivalent thereof.

2. A method according to claim 1, wherein said alpha2,6 sialyltransferase is human alpha2,6 sialytransferase.

3. A method for producing a virus particle, said method comprising the steps of:
   - contacting a cell with a virus particle in a culture medium under conditions conducive to infection of said cell by said virus particle; and
   - culturing said cell under conditions conducive to propagation of said virus particle,

   wherein said cell over-expresses a nucleic acid encoding an alpha2,3 sialyltransferase or a functional equivalent thereof.

4. A method according to claim 3, wherein said alpha2,3 sialyltransferase is human alpha2,3 sialytransferase.

5. A method according to any one of claims 1-4, wherein said virus particle is an influenza virus particle or an equivalent thereof.

6. A method according to claim 5, wherein said influenza virus particle is present in an influenza isolate.

7. A method according to claim 6, wherein said influenza isolate is obtained from at least one influenza-infected mammalian subject.

8. A method according to claim 7, wherein said influenza-infected mammalian subject is human or pig.

9. A method according to claim 6, wherein said influenza isolate is obtained from at least one influenza-infected bird.

10. A method according to any one of claims 1-9, wherein said cell is transformed with E1 from adenovirus.

11. A method according to claim 10, wherein said cell is a human cell.

12. A method according to claim 11, wherein said human cell is PER.C6 or a derivative thereof.

13. A method according to any one of claims 1-12, wherein said nucleic acid encoding the sialyltransferase is heterologous to said cell.

14. A method according to claim 13, wherein said nucleic acid encoding the sialyltransferase or functional equivalent is integrated into the genome of said cell.

15. A method for making a vaccine, said method comprising the steps of:

- producing a virus particle according to any one of claims 1-14; and
- inactivating the virus particles so produced.

16. A method according to claim 15, wherein said method further comprises the steps of:
    - treating said virus particles so produced to yield antigenic parts; and
    - obtaining at least one antigenic part.

17. A method according to claim 16, wherein said antigenic part comprises the hemagglutinin protein or a part thereof, and/or the neuraminidase protein or a part thereof, from influenza virus.

18. A vaccine obtainable according to any one of claims 15-17.

19. A pharmaceutical composition comprising a vaccine according to claim 18.

20. Use of a cell that over-expresses an alpha2,6 sialyltransferase or a functional part thereof for the propagation of a virus particle.

21. Use of a cell that over-expresses an alpha2,3 sialyltransferase or a functional part thereof for the production of a virus particle.

22. Use of a cell according to claim 20 or 21, wherein said virus particle is an influenza virus particle.

23. Use according to claim 22, wherein said influenza virus particle is present in an influenza isolate obtained from at least one influenza-infected mammalian subject.

24. Use according to claim 23, wherein said influenza-infected mammalian subject is a human or a pig.

25. Use according to claim 22, wherein said influenza virus particle is present in an influenza isolate obtained from at least one influenza-infected bird.

26. A method for selective propagation of a set of predetermined virus particles present in an isolate, wherein said set of predetermined virus particles has affinity for a specific glycosylation moiety present on a receptor, and wherein said isolate comprises in addition to said set also virus particles not having the predetermined specificity, said method comprising the steps of:

- incubating a cell which is capable of expressing and exposing said receptor comprising said specific glycosylation moiety, with said isolate in a culture medium under conditions conducive to infection of said cell by at least one virus particle present in said set;
- culturing said cell under conditions conducive to propagation of said virus particle; and
- harvesting virus particles so produced from said cell and/or said culture medium.

27. A method according to claim 26, wherein said glycosylation moiety comprises a SAalpha2,6Gal residue.

28. A method according to claim 26, wherein said glycosylation moiety comprises a SAalpha2,3Gal residue.

29. A method according to any one of claims 26-28, wherein said set of predetermined virus particles is a set of predetermined influenza virus particles.

30. A method according to any one of claims 26-29, wherein said isolate is an influenza isolate.

31. A method according to claim 30, wherein said influenza isolate is obtained from at least one influenza-infected human, pig or bird,

32. A method according to any one of claims 26-31, wherein said cell is transformed with E1 from adenovirus.

33. A method according to claim 32, wherein said cell is a human cell.

34. A method according to claim 33, wherein said human cell is PER.C6 or a derivative thereof.

35. A method according to any one of claims 26-34, wherein said cell comprises a nucleic acid encoding a sialyltransferase that is heterologous to said cell.

36. A method according to claim 35, wherein said nucleic acid encoding a sialyltransferase is integrated into the genome of said cell.

37. A human cell comprising a heterologous nucleic acid encoding an alpha2,6 sialyltransferase.

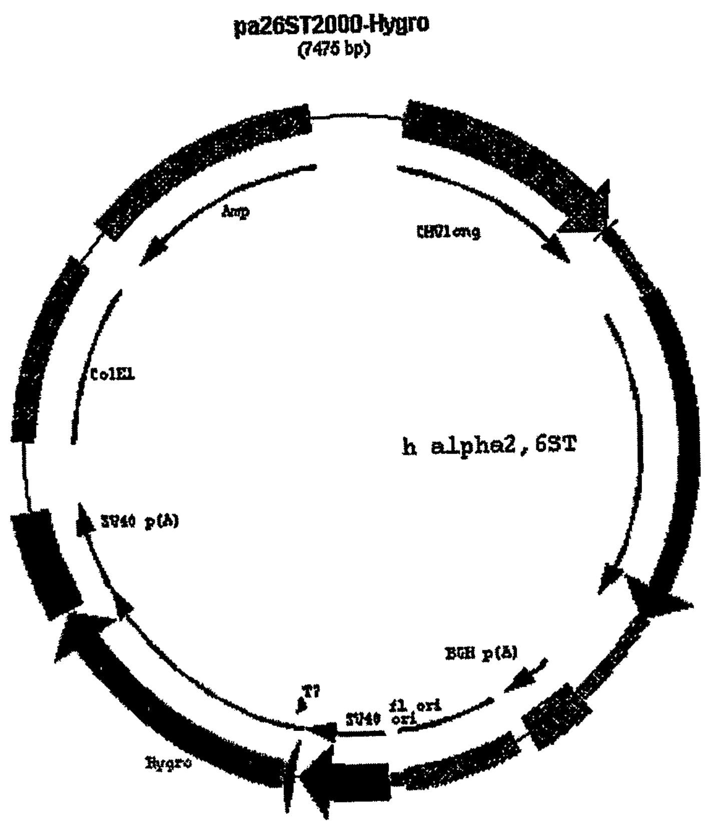
38. A human cell comprising a heterologous nucleic acid encoding an alpha2,3 sialyltransferase.

39. A human cell according to claim 37 or 38, wherein said heterologous nucleic acid is integrated into the genome of said human cell.

40. Use of a human cell according to any one of claims 37-39 for the selective propagation of a virus particle.

41. Use of a human cell according to claim 40, wherein said virus particle is an influenza virus particle.

Figure 1.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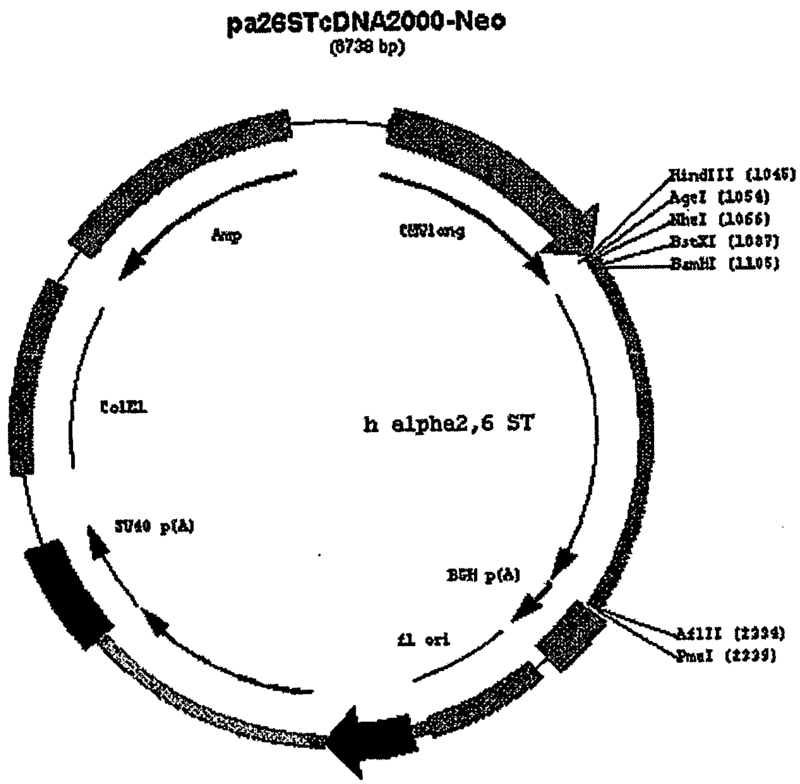
Hygro

Figure 2A.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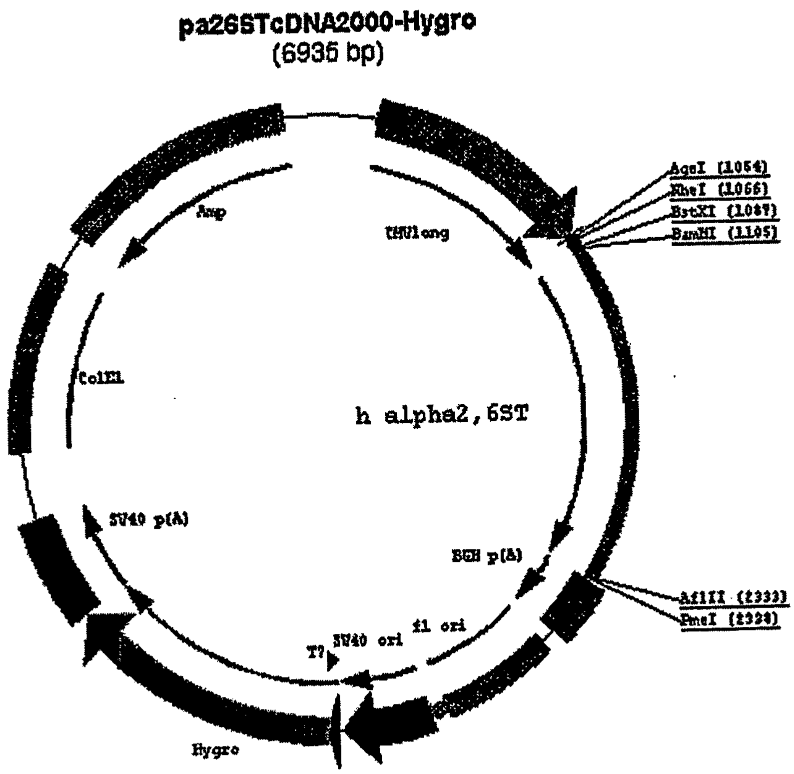
PmeI (2339)

Figure 2B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h alpha2,6ST
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SV40 ori
f1 ori
Hygro

Figure 3A

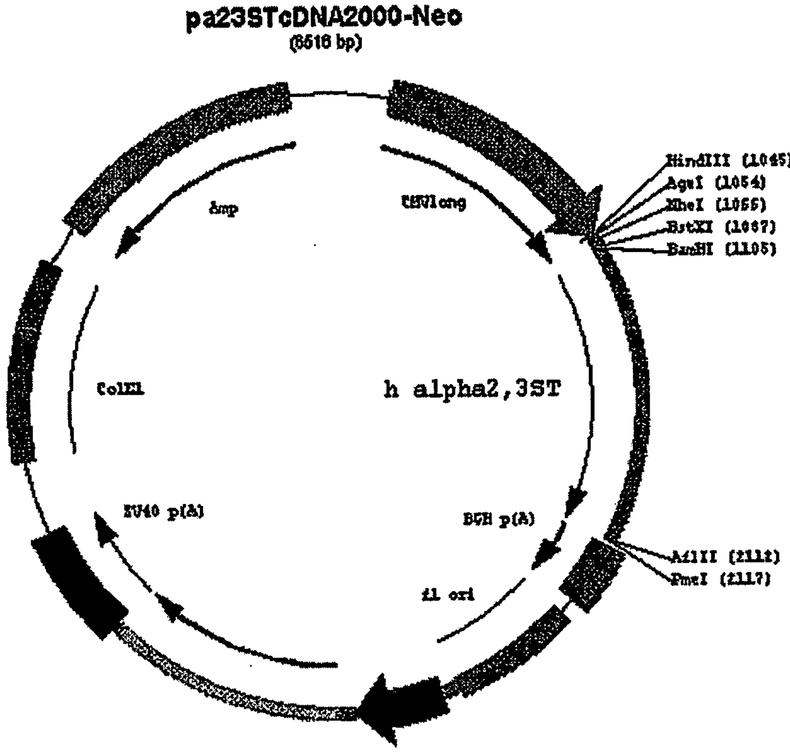
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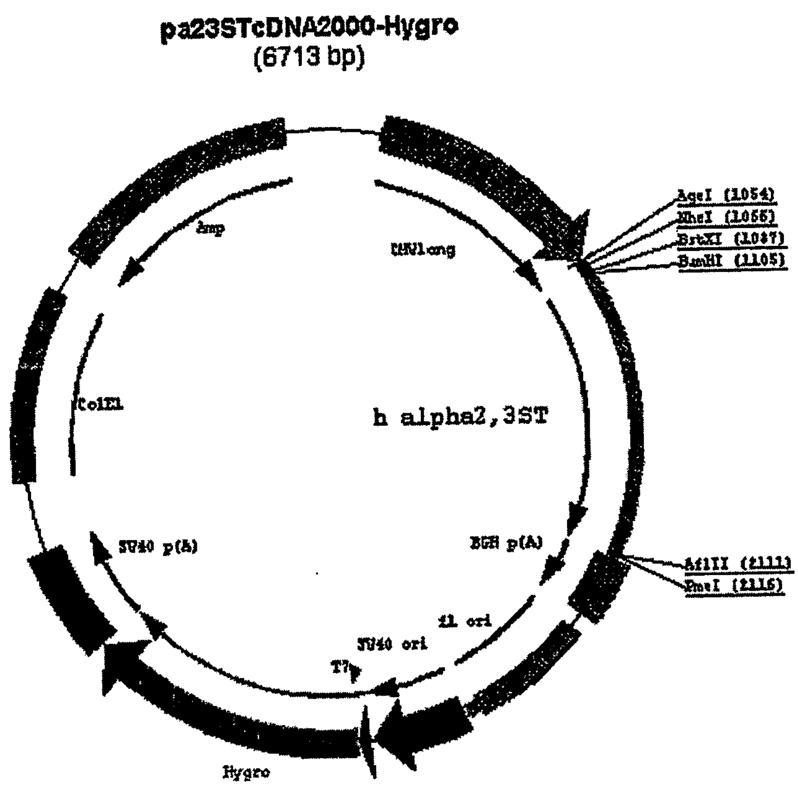
Figure 3B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h alpha2,3ST
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SV40 ori
T7
Hygro

Figure 4

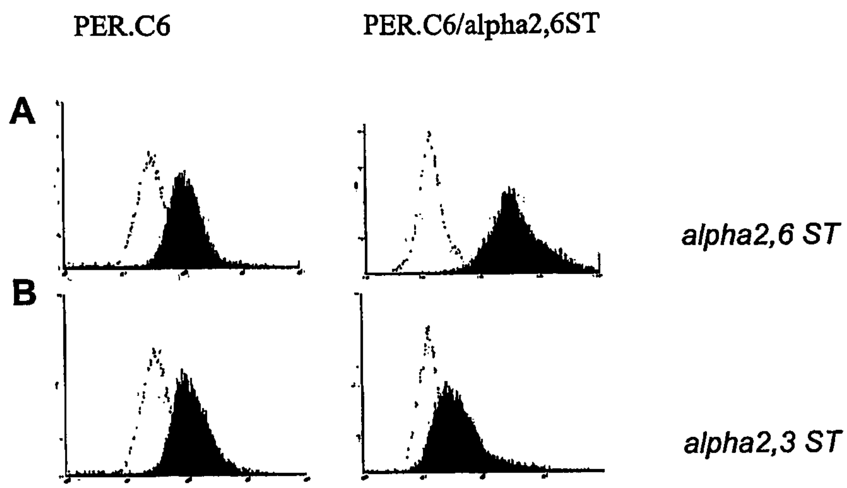


Figure 5

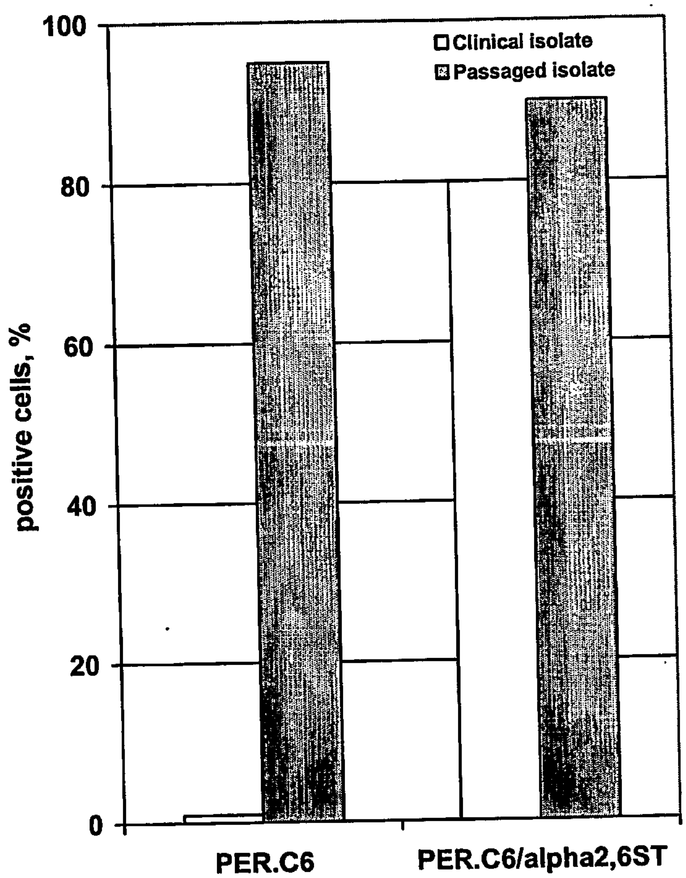
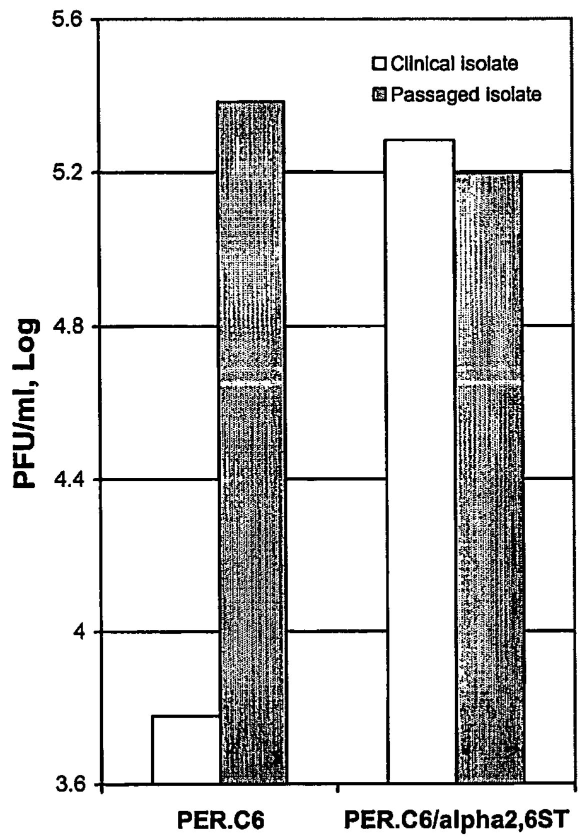

100
80
60
40
20
0
positive cells, %
Clinical isolate
Passaged isolate
PER.C6
PER.C6/alpha2,6ST

Figure 6.


5.6
5.2
4.8
4.4
4
3.6
PFU/ml, Log
Clinical isolate
Passaged isolate
PER.C6
PER.C6/alpha2,6ST

INTERNATIONAL SEARCH REPORT

PCT/NL 01/00892

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/11 C12N5/10 C12N7/00 A61K39/145 A61P31/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH, BIOTECHNOLOGY ABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 38362 A (CRUCELL HOLLAND B V ;SCHOUTEN GOVERT JOHAN (NL); PAU MARIA GRAZIA) 31 May 2001 (2001-05-31) cited in the application page 9, line 1 -page 14, line 11 page 18, line 1 - line 4 page 22, line 11 -page 24, line 7 page 28, line 28 -page 29, line 7	26-34
A	claims 1-45	1-25, 35-41

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
29 July 2002	09/08/2002

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	PAU M G ET AL: "The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 19, no. 17-19, 2001, pages 2716-2721, XP002201217 ISSN: 0264-410X cited in the application the whole document especially page 2721 right-handed column lines 3-13 the whole document ---	26-34
X	CARROLL S M ET AL: "DIFFERENTIAL INFECTION OF RECEPTOR-MODIFIED HOST CELLS BY RECEPTOR-SPECIFIC INFLUENZA VIRUSES" VIRUS RESEARCH, vol. 3, no. 2, 1985, pages 165-180, XP001095308 ISSN: 0168-1702 the whole document ---	26-31
X	WO 00 63403 A (SCHOUTEN GOVERT JOHAN ;HATEBOER GUUS (NL); BOUT ABRAHAM (NL); INTR) 26 October 2000 (2000-10-26) cited in the application page 20, line 15 -page 23, line 27 examples 16,27,28 claims 1-12,40-81 -----	37-39

INTERNATIONAL SEARCH REPORT

PCT/NL 01/00892

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